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<p>(21) International Application Number: PCT/US96/20341 (22) International Filing Date: 27 December 1996 (27.12.96) (30) Priority Data: 60/009,432 29 December 1995 (29.12.95) US (71) Applicant (for all designated States except US): A AND D ASSAY, INCORPORATED [US/US]; 31 Bishop Lane, Sudbury, MA 01776 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): HOLICK, Michael, F. [US/US]; 31 Bishop Lane, Sudbury, MA 01776 (US). RAY, Rahul [US/US]; 12 Gray Birch Lane, Wayland, MA 01778 (US). (74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).</p>		<p>(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i></p>
<p>(54) Title: LABELED VITAMIN D COMPOUNDS AND THE USE THEREOF (57) Abstract <p>Biotin, fluorescent and chemiluminescent labeled vitamin D compounds are disclosed as well as their use in assays for the presence of vitamin D, its metabolites and vitamin D analogs in biological fluids.</p></p>		

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Labeled Vitamin D Compounds and the Use Thereof

Background of the Invention

Field of the Invention

5 The present invention relates to non-radioactive vitamin D compounds and methods to assay for the presence of vitamin D, vitamin D analogs and their metabolites which may be present in milk, blood or other biological fluids. The assay methods employed in this invention may be enzyme linked immunoassays (ELISAs) (with biotin containing compounds) and fluorimetric and chemiluminometric assays (with fluorescein or chemiluminescence containing
10 compounds).

Related Art

15 It is well-established that cutaneously synthesized vitamin D₃, a seco-steroid, undergoes sequential metabolic conversions to 25-hydroxyvitamin D₃ (25-OH-D₃) in the liver and to 1,25(OH)₂D₃ in the kidney. 1,25(OH)₂D₃, the dihydroxylated metabolite of vitamin D₃, is the most active form of vitamin D hormone which is intimately involved in calcium and phosphorous homeostasis (Holick, M.F. (1989), "Vitamin D: biosynthesis, metabolism and mode of action." In *Endocrinology*, vol. 2, Degroot *et al.* (eds.), Saunders, W.B., Philadelphia, pp. 902-926). In addition to vitamin D₃ (synthesized in the skin), another chemical
20 form of vitamin D₃, called vitamin D₂, exists in nature. Vitamin D₂ is metabolized to 25-hydroxyvitamin D₂ (25-(OH)₂-D) and 1,25(OH)₂D₂ in a manner similar to vitamin D₃. Vitamin D₂ is obtained primarily from diet and vitamin D supplementation, and can be as little as 5-10%, or as high as 100% of the circulating concentration of 25-OH-D depending on the relative amounts of
25 vitamin D₂ present in the diet and cutaneously-produced vitamin D₃ by exposure to sunlight (Holick, M.F. *et al.* (1986) "Calcium, phosphorus and bone metabolism: calcium regulating hormones," in *Harrison's Principles of Internal*

Medicine, 13th Ed., Braunwald *et al.* (eds.), McGraw-Hill, New York, pp. 2137-2151). In the following discussion, it may be assumed that vitamin D, 25-OH-D and 1,25(OH)₂D will represent the total pool of vitamin D and its metabolites, unless otherwise mentioned.

5 Biosynthesis of 25-OH-D and 1,25(OH)₂D and their metabolism are regulated by the factors that control mineral and skeletal metabolism (Holick, M.F. (1989)). As a result, the serum 1,25(OH)₂D level is an important pathophysiological indicator in several diseases. For example, production of 1,25(OH)₂D is strongly influenced by a number of diseases such as acquired or
10 inherited disorders of vitamin D-metabolism, including renal osteodystrophy, certain metabolic bone diseases, sarcoidosis, hypercalcemia associated with chronic granulomatous disorders, and vitamin D-dependent rickets types I and II (Holick, M.F. *et al.* (1986)).

 On the other hand, the circulating concentration of 25-OH-D is considered
15 to be an important indicator of vitamin D status in man (Holick, M.F. (1989); Holick, M.F. *et al.* (1986)). For example, hypovitaminosis, which results from the insufficient endogenous production of vitamin D in the skin, and insufficient dietary supplementation, and/or inability of the small intestine to absorb adequate amounts of vitamin D from diet, results in hypocalcemia and hypophosphatemia
20 and corresponding secondary hyperparathyroidism (Holick, M.F. *et al.* (1986)). Vitamin D-deficiency is best determined in a clinical setting by measuring 25-OH-D in blood. When the 25-OH-D concentration is below the low limit of the normal range, the patient is considered to be deficient in vitamin D. Hypovitaminosis D also results in disturbances in mineral metabolism (i.e.,
25 rickets and osteomalacia in children and adults, respectively).

 Serum 25-OH-D-levels are also found to be lower than normal in intestinal malabsorption syndromes, liver disorders (chronic and acute), and nephrotic syndromes. In osteopenia in the aged, serum 25-OH-D levels are often found to be lower than normal. In cases of vitamin D intoxication, serum

25-OH-D level is found, as expected, to be higher than normal (Holick, M.F. *et al.* (1986)).

Considerable efforts have been directed towards developing assays for accurately measuring concentrations of 25-OH-D in circulation, given its pathophysiological importance. The 25-OH-D assays have been developed for measuring vitamin D status, especially in the elderly and patients with liver diseases and intestinal disorders.

The most efficient methods for assaying 25-OH-D known to date include different variations of a theme that involves competitive binding between 'cold' and 'hot' (radioactive) 25-OH-D₃ with normal or vitamin D-deficient rat serum (rat DBP). A standard curve is set up with measured quantities of 25-OH-D₃. An organic extract of a blood sample is added to the assay, and concentration of 25-OH-D is determined from the standard curve. Serum-concentration of 25-OH-D is much higher (on the order of 100-1000-fold) than the dihydroxylated metabolites of vitamin D, and hence these metabolites do not interfere with the assay in any significant way. This situation is further aided by higher binding avidity of DBP towards 25-OH-D compared with other dihydroxylated metabolites of vitamin D. Furthermore, DBP does not discriminate between 25-OH-D₂ and 25-OH-D₃, and hence the measured concentration of 25-OH-D in serum represents the total concentration of 25-OH-D₂ and 25-OH-D₃ (Chen *et al.*, *J. Nutritional Biochem.* 1:315-319 (1990)).

DeLuca, U.S. Patent No. 4,297,289, discloses vitamin D compounds isotopically labeled at the 6-position with deuterium or tritium atoms and the use thereof in vitamin D metabolite analyses.

DeLuca, U.S. Patent No. 4,816,417, discloses a competitive binding assay for the presence of 1,25(OH)₂D_x, where x is 2, 3, 4, 5 and/or 6, in a sample containing vitamin D transport protein. According to this assay, receptor protein which is capable of binding to 1,25(OH)₂D and labeled 1,25(OH)₂D is added to the sample together with an antibody capable of binding to the receptor protein.

One then measures the relative degree of binding of labeled 1,25-(OH)₂D to the receptor protein. The 1,25(OH)₂D is radiolabeled.

The above-mentioned assays, despite their specificity and efficiency, suffer from a few drawbacks. These assays are time-consuming and costly. The most important problem is, however, the intrinsic use of radioactivity. Radioisotopes are very costly, hazardous to handle and store. Radioactive disposal is also becoming an extremely costly affair.

An HPLC-UV detection method, which largely does not use radioactivity, has also been developed for assaying 25-OH-D (Jones, G., *Clin. Chem.* 24:287-298 (1978)). This method involves multiple chromatographic separations, and final detection and measurement of peaks corresponding to 25-OH-D₂ and 25-OH-D₃. Although this method provides one of the most accurate measurement of 25-OH-D in serum, it suffers from two major drawbacks. For example, measurement of 25-OH-D is limited by the detection limit of the UV detector. Therefore, 2 ml of blood is needed for the assay. This volume requirement is a particularly difficult problem for determining 25-OH-D levels in younger children. In addition, the assay procedure is very labor intensive and, therefore, very costly.

A non-radioactive method involving isotope-dilution mass spectrometry has also been developed. In this method, a serum sample is spiked with a synthetic analog of 25-OH-D₃ which is labeled with stable H-atoms (i.e., deuterium at C-26(27) (Bjorkhem and Holmberg, *Clin. Chim. Acta* 68:215-224 (1976)) or C6 and C-19 positions (Ray, R. *et al.*, *Steroids* 57:142-146 (1992)). The 'spiked' serum samples are processed in the usual fashion, i.e. extraction and partial purification of 25-OH-D fraction (by various chromatographic steps), and subjected to mass spectrometry. Concentration of metabolite in the serum is determined by the relative abundance of a particular "molecular fragment" (generated from the parent metabolite) compared with that of the labeled fragment. This method, although very accurate, has received little practical application due to the requirement of highly sophisticated and expensive

instrumentation. This method is also very time-consuming and costly. Furthermore, this method is specific for either 25-OH-D₃ or 25-OH-D₂, and hence underestimates the total concentration of circulating 25-OH-D.

5 In summary, 25-OH-D and 1,25(OH)₂D assays, known to date, suffer from various disadvantages, including long turn-around time, inter-assay variabilities, high cost and, in particular, the intrinsic use of radioactivity (in most assays), and are not best-suited for routine clinical assays. Furthermore, some of these assays (i.e., HPLC assay) require a fairly large volume of serum. This is a serious problem particularly in cases of neonates, infants and young children. Hence, there is an urgent need for a non-radioactive and highly sensitive method for assaying 25-OH-D and 1,25(OH)₂D in blood which will not be tedious, time-consuming and very expensive. The present invention overcomes these problems by providing a novel non-radioactive method for assaying 25-OH-D and 1,25(OH)₂D in blood that is rapid, relatively inexpensive and easy to perform.

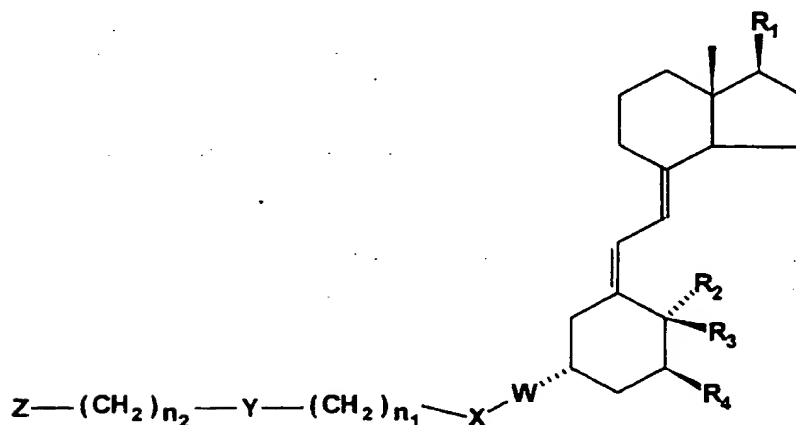
15 The measurement of 1,25(OH)₂D is very valuable in determining the etiology of inborn and acquired disorders of 25-OH-D metabolism (Holick (1989)). Therefore, the assay of 1,25(OH)₂D is valuable clinically and a new method that is rapid and does not use radioactivity is highly desirable.

20 A large number of active vitamin D compounds are known which are useful for various therapeutic purposes. See, for example, U.S. Patent Nos. 5,457,217, 5,414,098, 5,384,313, 5,373,004, 5,371,249, 5,430,196, 5,260,290, 5,393,749, 5,395,830, 5,250,523, 5,247,104, 5,397,775, 5,194,431, 5,281,731, 5,254,538, 5,232,836, 5,185,150, 5,321,018, 5,086,191, 5,036,061, 5,030,772, 5,246,925, 4,973,584, 5,354,744, 4,927,815, 4,857,518, 4,851,401, 4,851,400, 4,847,012, 4,755,329, 4,940,700, 4,619,920, 4,594,192, 4,588,716, 4,564,474, 25 4,552,698, 4,588,528, 4,719,204, 4,719,205, 4,689,180, 4,505,906, 4,769,181, 4,502,991, 4,481,198, 4,448,726, 4,448,721, 4,428,946, 4,411,833, 4,367,177, 4,336,193, 4,360,472, 4,360,471, 4,307,231, 4,307,025, 4,358,406, 4,305,880, 4,279,826, and 4,248,791.

Vitamin D content in milk is determined by a laborious assay (Holick *et al.*, *N. Eng. J. Med.* 132:1178-81 (1992), Tanner *et al.*, *J. Assoc. of Analyt. Chem.* 17:607-710 (1988)). Thus, there is a need for a rapid non-radioactive assay for vitamin D in milk, blood, body fluids, foods and animal feed. These non-radioactive assays can be used to detect the presence of therapeutically useful vitamin D compounds.

Summary of the Invention

The present invention relates to a compound having the formula:



10 wherein:

R_1 is a substituted or unsubstituted alkyl, alkenyl or alkynyl group having 1 to 15 C-atoms which may be substituted by one or more hydroxy, halo, lower alkoxy, oxo, oxime, lower alkanoyloxy, aryloxy, aryl, benzoyl, a C_4 lactone, a C_4 lactone substituted by a methyl and a hydroxy group, C_3 - C_6 cycloalkyl, or C_3 - C_6 cycloalkyl substituted by hydroxy, lower alkyl, or hydroxyloweralkyl;

15

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R₂ is a methyl group and R₃ is hydrogen, or

R₂ is hydrogen and R₃ is a methyl group, or

R₂ and R₃ are both hydrogen or,

R₂ and R₃ together are a methylene group (=CH₂),

5 R₄ is hydrogen, hydroxy, lower alkoxy or lower alkanoyloxy,

W is oxygen or amino;

X is carbonyl (C=O) or methylene (CH₂);

Y is oxygen, sulfur, amino -C(O)O- or -C(O)-NH-;

Z is biotin, a fluorescent group or a chemiluminescent group; and

10 n₁ and n₂ are independently 1, 2, 3, 4, or 5.

The invention also relates to an assay method for the presence of vitamin D compounds in a sample, the improvement comprising using as the labeled vitamin D compound, a labeled compound of the present invention.

15 The invention also relates to a solid phase assay method for detecting a vitamin D, its metabolite or analog, comprising

(a) providing a solid phase support having immobilized thereon a protein or antibody which is capable of binding to the labeled compound of the present invention;

20 (b) contacting said solid phase support with a solution of the labeled compound of the invention for a time sufficient to allow binding of the labeled compound to said protein or antibody;

(c) washing the solid phase support obtained in step (b) for a time sufficient to remove unbound labeled compound;

25 (d) contacting the solid phase support obtained in step (c) with a liquid sample suspected of obtaining a vitamin D, its metabolite or analog for a time sufficient to effect displacement of the labeled compound from said protein or antibody;

(e) removing the liquid obtained in step (d); and

30 (f) detecting the presence of the labeled compound in the liquid obtained in step (e);

wherein the amount of labeled compound detected in step (f) is directly proportional to the amount of the vitamin D, its metabolite or analog in said test sample.

Brief Description of the Figures

5 Fig. 1 depicts a competitive binding assay for 25-OH-D₃ biotin and fluorescein conjugates.

Fig. 2 depicts a schematic representation of the assay of the invention.

Fig. 3 depicts a vitamin D assay flow diagram for milk.

10 Fig. 4 depicts assays for vitamin D₃ and 25-OH-D₃ using the double-antibody technique.

Fig. 5 depicts assays for vitamin D₃ and 25-OH-D₃ using the avidin-biotin chemistry technique.

Fig. 6 depicts a scheme for the preparation of a biotin conjugate of 1,25(OH₂)D₃.

Detailed Description of the Preferred Embodiments

With respect to the formula above:

Typical C₆₋₁₄ aryl groups include phenyl, naphthyl, phenanthryl, anthracyl, indenyl, azulenyl, biphenyl, biphenylenyl and fluorenyl groups.

Typical halo groups include fluorine, chlorine, bromine and iodine.

20 Typical C₁₋₁₅ alkyl groups include methyl, ethyl, propyl, isopropyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl groups as well as branched chain alkyl groups. Preferably, when R₁ is an alkyl group, it is the C-17 side chain of vitamin D₂ or D₃.

25 Typical C₂₋₁₅ alkenyl groups include ethenyl, propenyl, butenyl, pentenyl, hexenyl, heptenyl, octenyl, nonenyl, decenyl groups and the like as well as the

branched chain alkenyl groups. Preferably, when R_1 is an alkenyl group, it is the C-17 side chain of vitamin D_2 or D_3 with a double bond at C22-23.

Typical C_{2-4} alkynyl groups include ethynyl, propynyl, butynyl, pentynyl, hexynyl, heptynyl, octynyl, nonynyl, decynyl groups and the like as well as the branched chain alkynyl groups. Preferably, when R_1 is an alkynyl group, it is the C-17 side chain of vitamin D_3 with a triple bond at C22-23.

Typical lower alkoxy groups include oxygen substituted by one of the C_{1-4} alkyl groups mentioned above.

Typical lower alkanoyloxy groups include any C_{1-6} acyloxy groups, e.g. acetoxy, propanoyloxy, butanoyloxy, pentanoyloxy, hexanoyloxy and the like.

Typical aryloxy groups include oxygen substituted by any one of the aryl groups mentioned above.

Preferably, R_1 is a side chain of vitamin D_2 (carbon positions C_{20} - C_{27}) or D_3 (carbon positions C_{20} - C_{27}), or these chains partially modified with one or more hydroxy groups on C_{23} , C_{24} and/or C_{25} .

Preferably, Z is biotin, fluorescein or any of their natural or synthetic derivatives or any other synthetic or natural derivatives which are ascribed to function as biotin or fluorescein (e.g., (4,4-difluoro-4-bora-3a,4a-diaza-5-indacene (BODIPY), rhodamine, phycoertherin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine). Z may also be a chemiluminescent molecule such as luminol, isoluminol, thermomatic acridium ester, imidazole, acridinium salt and 1,2-dioxetanes (see U.S. Patent Nos. 4,931,569, 4,959,182, 5,004,565, 4,857,652, and 4,962,192).

The label may instead be attached at the 1-position of the vitamin D compound. In this embodiment, R_4 is the C_3 substituent depicted in the formula above, and there is a hydroxy group at the 3beta position.

Preferred labeled compounds include 25-hydroxy-3-aminopropoxyvitamin D_3 biotinamide, 25-hydroxy-3beta-[(5-biotinamidyl)pentanamido]-3-aminopropoxyvitamin D_3 , 25-hydroxy-3beta-[(6-biotinamidyl)-hexanamido]-3-

aminopropoxyvitamin D₃, 25-hydroxy-3beta-aminopropoxyvitamin D₃ fluorescein amide, 1,25-dihydroxy-3-aminopropoxyvitamin D₃ biotinamide, 1,25-dihydroxy-3beta-[(5-biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃, 1,25-dihydroxy-3beta-[(6-biotinamidyl)-hexanamido]-3-aminopropoxyvitamin D₃, 1,25-dihydroxy-3beta-aminopropoxyvitamin D₃ fluorescein amide, 3-aminopropoxyvitamin D₃ biotinamide, 3beta-[(5-biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃, 3beta-[(6-biotinamidyl)-hexanamido]-3-aminopropoxyvitamin D₃, and 3beta-aminopropoxyvitamin D₃ fluorescein amide.

The labeled compounds of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therein one or more container means such as vials, tubes, plates and the like, each of the container means comprising the separate components of the assay. For example, there may be a container means containing the labeled vitamin D compound of the invention, optionally in solution or bound to the walls of the container. Further container means may contain, for example, avidin coated beads, plates or tubes; proteins which bind vitamin D compounds, their metabolites and/or analogs; DBP; enzyme labeled antibodies, vitamin D binding protein or vitamin D receptor and their substrates; and/or buffers such as phosphate buffer saline (PBS) or bovine serum albumin (BSA).

The labeled compounds of the present invention may be used in any conventional assay for vitamin D compound, their metabolites and for vitamin D analogs that may be administered to a patient. Such assays are competitive binding assays and enzyme linked immunoassays (ELISAs). See, for example, Chen *et al.*, *J. Nutr. Biochem.* 1:272-276 (1990); Chen *et al.*, *J. Nutr. Biochem.* 1:315-319 (1990); Chen *et al.*, *J. Nutr. Biochem.* 1:320-327 (1990); Engvall, V. *Meth. Enzymol.* 70:419-439 (1980); Millipore Catalogue 1994-1995, Marlborough, MA; and U.S. Patent Nos. 4,297,289, 4,816,417, 5,232,836 and 4,585,741. The improvement which comprises the present invention is the

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substitution of the labeled vitamin D compounds of the present invention for the prior art radiolabeled compounds.

The invention also relates to a solid phase assay method for detecting a vitamin D, its metabolite or analog in a test sample, comprising

- 5 (a) providing a solid phase support having immobilized thereon a protein or antibody which is capable of binding to the labeled compound of the present invention;
- (b) contacting said solid phase support with a solution of the labeled compound of the invention for a time sufficient to allow binding of the labeled
10 compound to said protein or antibody;
- (c) washing the solid phase support obtained in step (b) for a time sufficient to remove unbound labeled compound;
- (d) contacting the solid phase support obtained in step (c) with a liquid test sample suspected of containing a vitamin D, its metabolite or analog for a
15 time sufficient to effect displacement of the labeled compound from said protein or antibody;
- (e) removing the liquid obtained in step (d); and
- (f) detecting the presence of the labeled compound in the liquid obtained in step (e);
20 wherein the amount of labeled compound detected in step (f) is directly proportional to the amount of the vitamin D, its metabolite or analog in said test sample.

Test samples which may be tested with the assay include extracts of animal feeds, foods containing vitamin D, milk, infant formula, blood, serum,
25 urine, saliva, peritoneal and pleural fluids as well as pills or medicaments that contain vitamin D or its metabolites or analogs.

Examples of solid phase supports include glass, plastic, nitrocellulose, diazocellulose, polystyrene, polyvinylchloride, polypropylene, polyethylene, dextran, Sepharose, agar, starch, nylon and microtitre plates.

Proteins which bind to the labeled vitamin D compounds include any such protein which is known. Typically, such proteins are receptor proteins which bind vitamin D, its metabolites and/or analogs. Preferred proteins are vitamin D binding protein (DBP), vitamin D receptor or avidin.

5 Alternatively, any antibody which is capable of binding vitamin D, its metabolite or analog can be used.

The solid support may be washed with any conventional buffer such as PBS and the like.

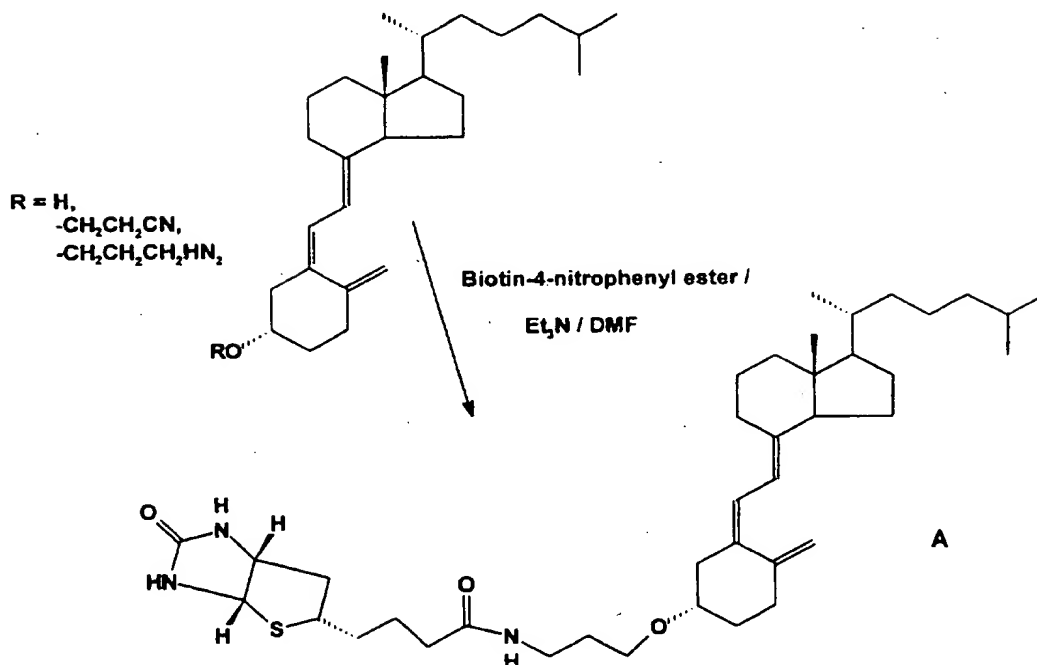
10 The label can be detected by any means known, for example, by visual inspection, fluorometric or spectrophotometric means. Methods for detecting such labels in solid phase assays are disclosed in U.S. Patent 5,098,846.

The labeled compounds of the present invention can be prepared according to the examples which follow. The starting vitamin D compounds and vitamin D analogs can be obtained according to the methods disclosed in U.S.
15 Patent Nos. 5,457,217, 5,414,098, 5,384,313, 5,373,004, 5,371,249, 5,430,196, 5,260,290, 5,393,749, 5,532,018, 5,086,191, 5,036,061, 5,030,772, 5,246,925, 4,973,584, 5,354,744, 4,927,815, 4,857,518, 4,851,401, 4,851,400, 4,847,012, 4,755,329, 4,940,700, 4,619,920, 4,594,192, 4,588,716, 4,564,474, 4,552,698, 4,588,528, 4,719,204, 4,719,205, 4,689,180, 4,505,906, 4,769,181, 4,502,991,
20 4,481,198, 4,448,726, 4,448,721, 4,428,946, 4,411,833, 4,367,177, 4,336,193, 4,360,472, 4,360,471, 4,307,231, 4,307,025, 4,358,406, 4,305,880, 4,279,826, and 4,248,791.

The following examples are illustrative, but not limiting, of the method and compositions of the present invention. Other suitable modifications and
25 adaptations of the variety of conditions and parameters normally encountered in clinical therapy and which are obvious to those skilled in the art are within the spirit and scope of the invention.

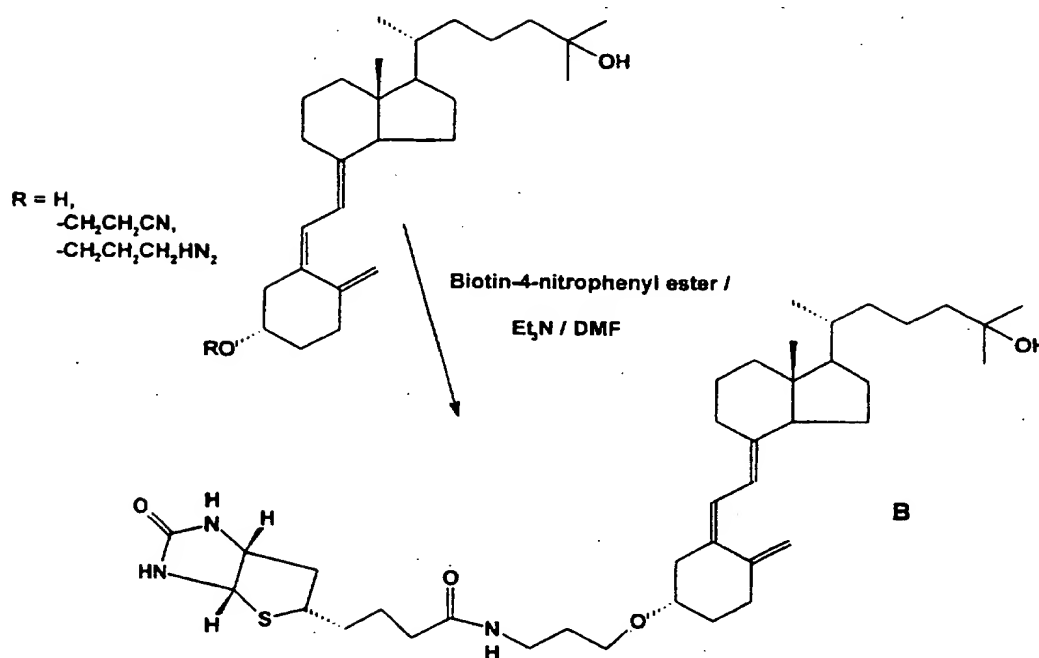
Examples

Example 1 Biotin conjugate of vitamin D₃-3-amino propyl ether (A)



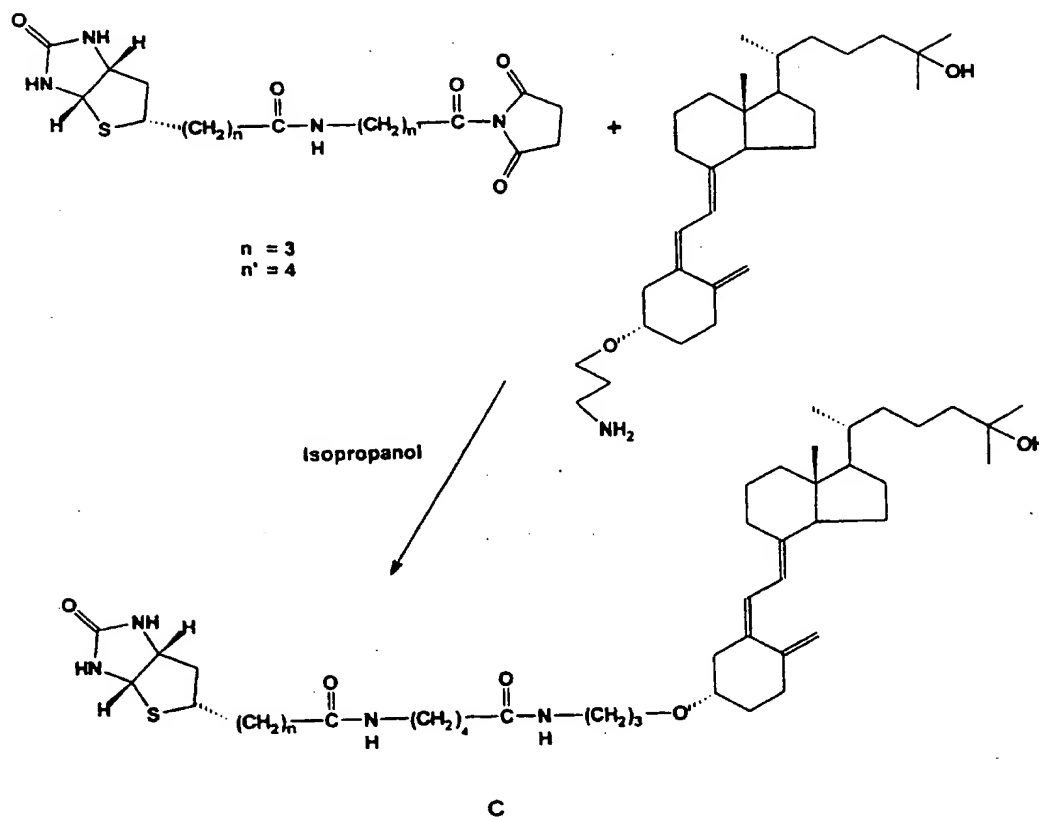
5 Compound A was synthesized by the reaction of vitamin D₃-3-aminopropyl ether with biotin-4-nitrophenyl ester in dimethyl formamide (DMF) in the presence of triethylamine (Et_3N). The product was purified by preparative TLC. Yield of A was 70%. NMR spectrum of A supported its structure. Related compounds of the invention may be prepared by reaction of the corresponding vitamin D₃-aminopropyl ether.

Example 2 *Biotin Conjugate of 25-Hydroxyvitamin D₃-3-aminopropyl Ether (B)*



Compound **B** was synthesized by the reaction of
5 25-OH-D₃-3-aminopropyl ether with biotin-4-nitrophenyl ester (Aldrich
Chemical Co.) in DMF in the presence of Et₃N. The product was purified by the
preparative TLC on silica gel (10% methanol in methylene chloride) in 45 %
yield. The adduct **B** had a UV spectrum having λ_{max} at 265 nm. Spectrum of **B**
was confirmed by NMR.

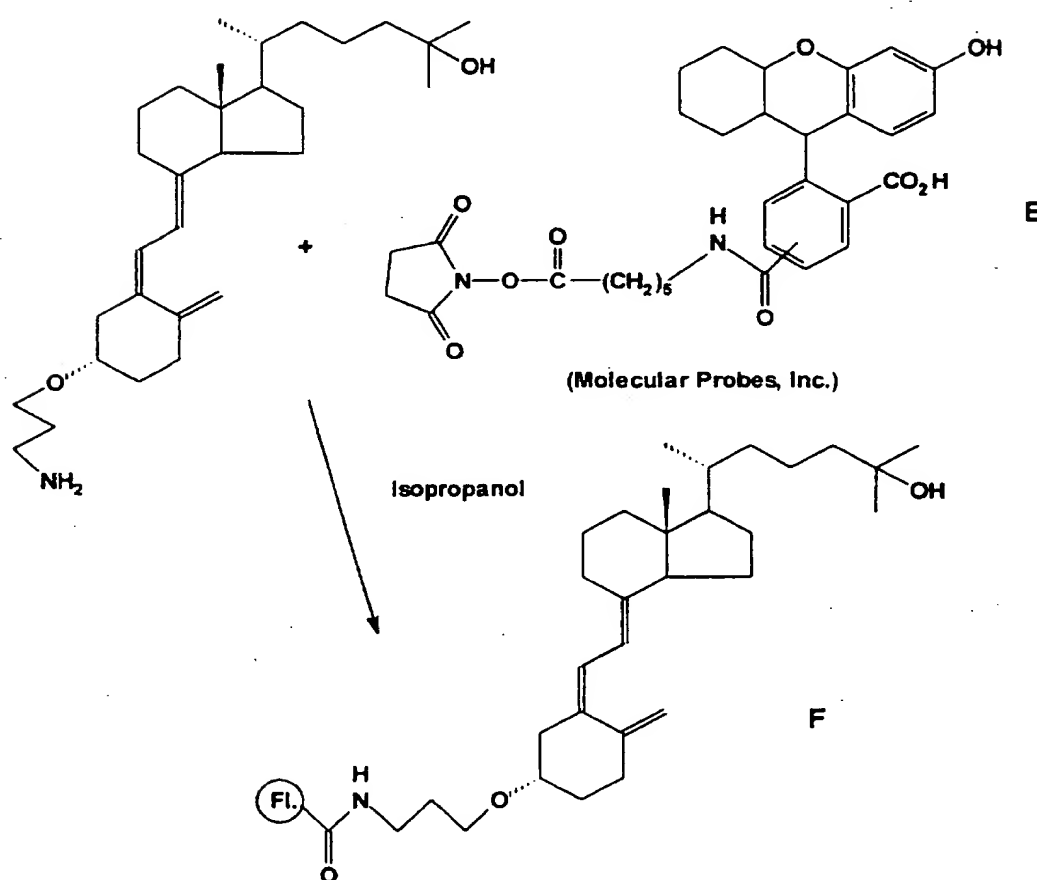
Example 3 Conjugate of 25-OH-D₃-3-aminopropyl ether and Biotin-X-NHS (C)



5 The solution of 25-OH-D₃-aminopropyl ether and biotin-X-NHS (Calbiochem Inc., San Diego CA) in isopropanol was stirred for one hour followed by the addition of a small amount of n-butylamine for dissipating the unreacted biotin-X-NHS. After an hour of stirring the solution was dried under argon and the reaction mixture was purified by preparative TLC (10% methanol in CH₂Cl₂). There were primarily two UV-active bands. The polar band was

10 further purified by HPLC (C₁₈ column, 5% water in MeOH). UV spectrum of compound C in methanol had a vitamin D-like spectrum with λ_{\max} at 265 nm and a λ_{\min} at 228 nm. NMR spectrum of C was consistent with its structure.

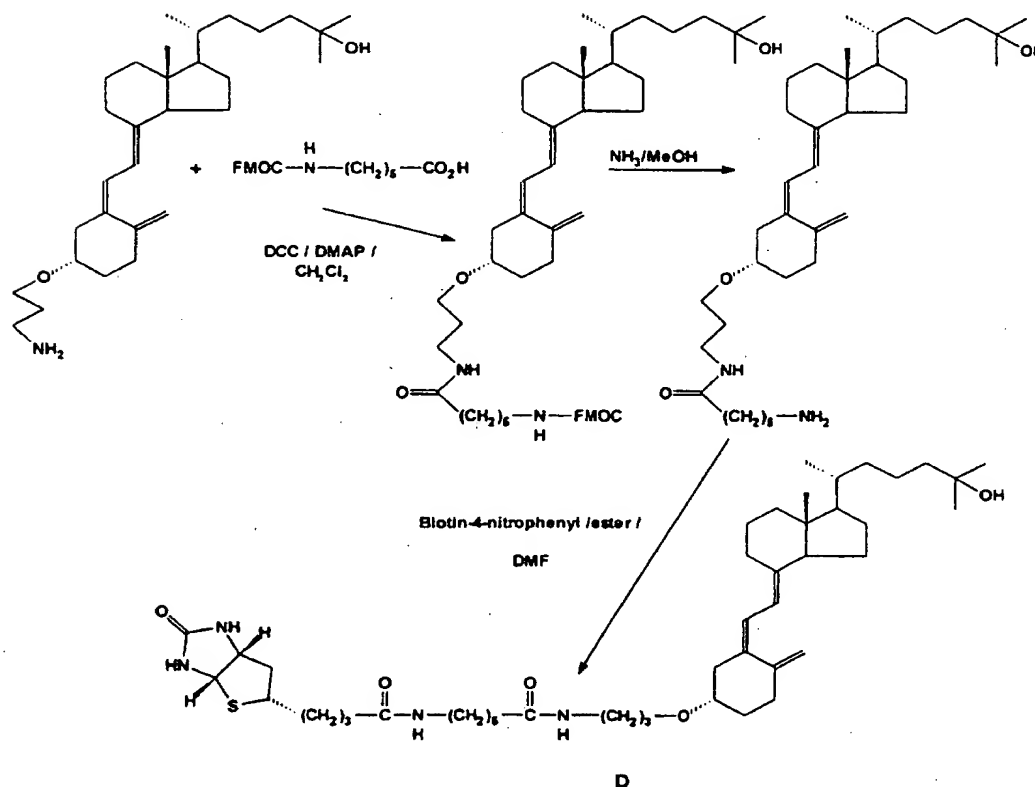
Example 4 *Synthesis of a conjugate of 25-OH-D₃-3-aminopropyl ether and a Fluorescein Derivative (F)*



A solution of fluorescein compound E (6-(fluorescein-5-(and
 5 6-)-carboxamido)hexanonic acid succinyl ester, Molecular Probes, Inc., Eugene,
 Or) and an excess of the 25-OH-D₃-3-aminopropyl ether in isopropanol was
 stirred for an hour followed by the removal of solvent. TLC of this reaction
 mixture (acetic acid: acetone: methanol:benzene - 0.25:0.25: 0.25:0.5:4.0)
 indicated the formation of a strongly fluorescent compound. It was isolated by
 10 preparative TLC using the same eluant.

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Example 5 *Biotin conjugate of 25-OH-D₃-3-aminopropyl-3-(6-amino)hexanoic acid (D)*



5 The compound **D** was synthesized according to this scheme. The product was characterized by UV (methanol), which had a λ_{max} of 265 nm, typical for vitamin D-triene system. NMR spectrum of **D**: δ 4.8 and 5.1 (AB_q pattern, exocyclic CH₂, 2H), δ 5.9 and 6.2 (double doublet, 2H, CH₂ olefinic proton).

Example 6 *Competitive radioligand binding assays of analogs (C) and (F) with human vitamin D-binding protein (hDBP)*

These assays were carried out to determine the viability of these synthetic analogs to serve as substrates for hDBP. It was observed that, on a molar basis, compound C was approximately eleven (11) times less efficient in displacing ^3H -25-OH- D_3 , bound to hDBP, than 25-OH- D_3 . Compound F was similar to 25-OH- D_3 in displacing ^3H -25-OH- D_3 (Fig. 1). The results shown in Fig. 1 demonstrate that compound C and D can replace 25-OH- D_3 for DBP binding.

Example 7 *Development of an enzyme-linked immunosorption assay (ELISA) for measuring the concentration of vitamin D_3 and 25-OH- D_3 in a test solution*

The method involves attachment of DBP to an ELISA plate followed by the addition of a known quantity of 25-OH- D_3 -biotin conjugate (compound C). This compound gets bound to DBP (attached to the plate) due to strong binding affinity between DBP and 25-OH- D_3 . Excess amount of C is washed off, and an excess of avidin is added which gets bound to biotin attached to 25-OH- D_3 . The plate is then incubated in sequence with anti-avidin antibody, a secondary antibody coupled to horseradish peroxidase (HRP), and ABTS (2,2'-azino-di-(3-ethylbenzthiazoline)sulfonate, the substrate that produces a blue color which may be monitored spectrophotometrically or visually. The intensity (absorbance) of the color is directly proportional to the concentration of compound C. In the first experiment, the optimal concentrations of DBP and compound C to develop color were determined (visually). In this experiment, each sample of DBP was tested against each amount of compound C.

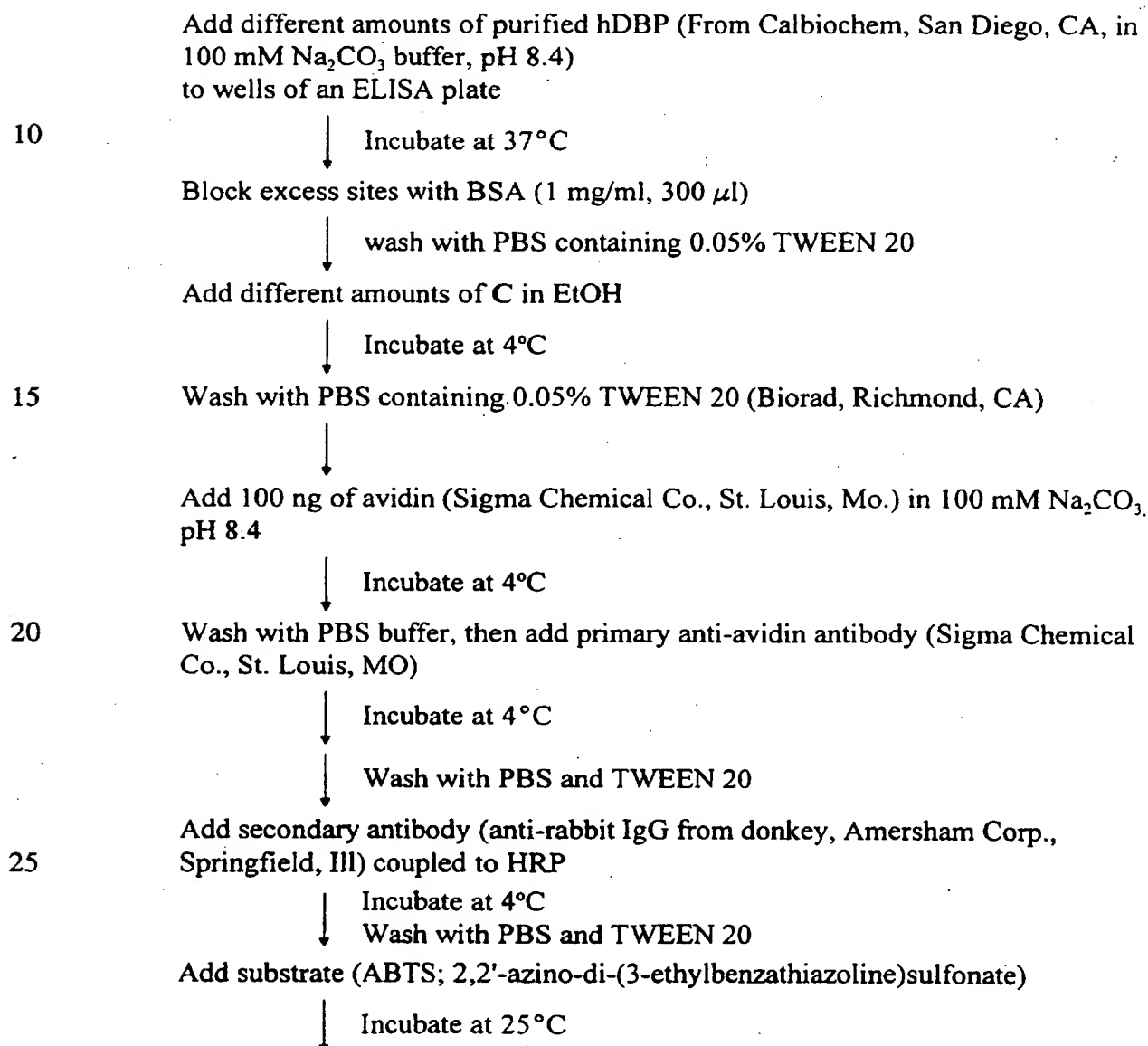
-19-

A. Determination of the optimal amount of DBP and compound (C) in a checkerboard titration (ELISA) to obtain a colorimetric signal

DBP used: 200, 100, 50, 25, 12.5, 6.25, 3.125 ng

C used: 1, 0.5, 0.25, 0.125, 0.066, 0.033, 0.016 μ g

Flowchart



Color development detected visually or spectrophotometrically.

Results: minimum amounts of DBP and compounds (C) required to obtain yellow color were 6.25 ng and 30 ng, respectively.

In the next experiment, it was determined whether it would be possible to displace compound C, bound to DBP (which is attached to an ELISA plate), with a known concentration of 25-OH-D₃. In a real sample, one will be measuring the amount of 25-OH-D and not that of compound C.

B. Assays to demonstrate that compound C can be competed out with 25-OH-D₃

Three concentrations of DBP/C were chosen.

	DBP / C	25-OH-D ₃
1	6.25 ng/30 ng	2 µg
2	12.5 ng/60 ng	2 µg
3	25 ng/120 ng	2 µg

Procedure: same as before, except either C, or C and 2 µg of 25-OH-D₃ were added to each plate. Each concentration (point) was done in triplicate. The blank contained no 25-OH-D₃ or C.

Color was absent with all the samples containing 25-OH-D₃ demonstrating that 25-OH-D₃ is capable of displacing compound C bound to DBP on an ELISA plate. Furthermore, the amount of compound C is directly proportional to the concentration of added 25-OH-D₃. Hence, the amount of displaced compound C can be used as a measure of 25-OH-D₃ in the assay system.

An alternate method to measure vitamin D or 25-OH-D with high sensitivity is as follows.

Flowchart

1. Coat the wells of a micro-titer plate with 200 ng of DBP, 2 hours at 37°C
2. Block excess sites with BSA (mg/ml in PBS-TWEEN 20 buffer), 2 hrs at 37°C
- 5 3. Make a serial dilution of 25-OH-D₃ (36 pg to 8.76 ng) or vitamin D₃ (0.36 ng to 85 ng)
4. Add compound C (25-OH-D₃-biotin) (800 pg) either alone or with different concentrations of 25-OH-D₃ or vitamin D₃
5. Incubate at 4°C for 12 hours
- 10 6. Wash the plate and incubate with avidin (100 ng/well) for 2 hrs at 4°C
7. Wash the plate and incubate with antiavidin antibody (1:10,000 dilution) for 2 hrs at 4°C
8. Wash the plate and incubate with HRP-coupled secondary antibody (1:5,000 dilution) for 2 hrs at 4°C
- 15 9. Wash the plate and incubate with ABTS solution
10. Stop the reaction by adding 0.05 ml of 20% SDS solution
11. Read the OD of each well at 410 nm.

Using the above procedure vitamin D₃ and 25-OH-D₃ were detected in picomolar levels as shown in Figure 4.

20 ***Example 8 Assays for vitamin D₃, 25-OH-D₃ and 1,25(OH)₂D₃ using avidin-biotin chemistry***

Stepwise procedure for an alternative method to measure vitamin D₃, 25-OH-D₃ and 1,25(OH)₂D₃ using biotin labeled vitamin D compounds is elaborated in the following.

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Flowchart

1. Coat wells of a micro-titer plate with avidin (200 ng per well) in 100 mM sodium bicarbonate buffer, pH 8.4
2. Block excess sites with BSA (1 mg/ml in PBS containing 0.05% TWEEN 20) for 2 hrs at 37°C. Wash the wells
3. Couple DBP with horseradish peroxidase (DBP*) by standard protocol
4. Incubate DBP* (100 ng) with compound C (400 pg) in the presence and absence of different concentrations of vitamin D₃ (1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, 160.0, 320.0, and 640 ng) or 25-OH-D₃ (20.0, 40.0, 80.0, 160.0, 320.0, 640.0, and 1280.0 pg) or 1,25(OH)₂D₃ (1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, 160.0, 320.0, and 640 ng) in 50 mM Tris.HCl buffer, pH 8.3, 150 mM NaCl, 1.5 mM EDTA, 0.1% Triton X 100, in 1.5 ml microfuge tubes (total volume 0.1 ml) at 4°C overnight
5. Add DBP*/vitamin D₃ or DBP*/25-OH-D₃ or DBP*/1,25(OH)₂D₃ incubation mixtures to respective wells, allow to sit at 25°C for 60 minutes
6. Wash the wells
7. Add substrate (ABTS) to each well, wait till color develops (10-20 minutes)
8. Stop the reaction by adding 0.05 ml of 20% SDS
9. Read OD at 410 nm.

Results of the above assay using standard samples of vitamin D₃, 25-OH-D₃ and 1,25(OH)₂D₃ are shown in Figure 5.

**Example 9 Synthesis of a Biotin Conjugate of 1,25(OH)₂D₃
(1 α ,25-dihydroxyvitamin D₃-3 β -(6-amidobiotinyl)hexanoate, G)**

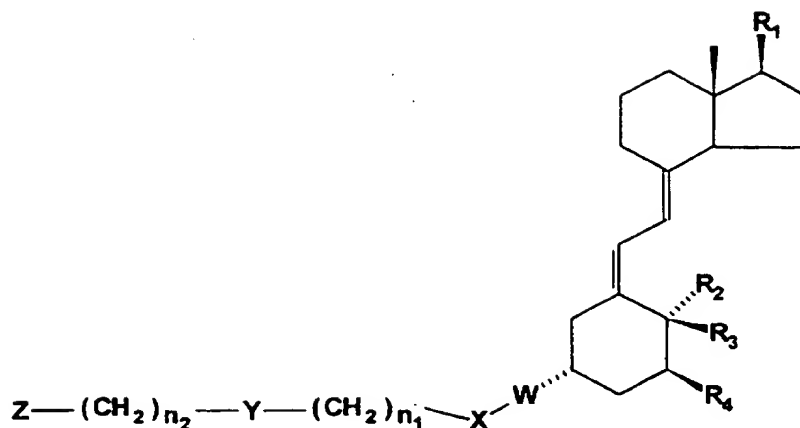
Assays for 1,25(OH)₂D a biotin conjugate of 1,25(OH)₂D₃ which binds to vitamin D receptor (VDR) are also desirable. A biotinylated derivative of 1,25(OH)₂D₃ was prepared as follows.

The title compound (G) was synthesized in a multi-step procedure (Fig. 6) starting with a derivative of 1,25(OH)₂D₃ in which the 1 α -OH group is protected as a tert. butyldimethylsilyl ether (TBDMS) (E) (Ray *et al.*, *J. Chem. Soc.*, 702-703 (1985), Ray *et al.*, *Biochem. Biophys. Res. Comm.* 132: 198-203 (1985), Ray and Holick, *Steroids* 51: 623-630 (1988), Ray *et al.*, *Steroids* 58: 462-465 (1993), Ray *et al.*, *Bioorganic Chem.* 22: 276-283 (1994), Ray *et al.*, *J. Biol. Chem.* 271: 2012-2017 (1996). DCC-coupling of compound E with Fmoc-caproic acid, followed by the removal of the 1-OH- and Fmoc-protecting groups produced the amine (F). Coupling of compound F with p-nitrophenyl ester of biotin produced the desired biotin conjugate of 1,25(OH)₂D₃ (compound G) in which biotin is attached to 1,25(OH)₂D₃ via a long tether. All the synthetic compounds were characterized by NMR and UV spectrometry.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions without undue experimentation. All patents and publications cited herein are incorporated by reference in their entirety.

What Is Claimed Is:

1. A compound having the formula:



wherein:

R_1 is a substituted or unsubstituted alkyl, alkenyl or alkynyl group having 1 to 15 C-atoms which may be substituted by one or more halo, hydroxy, lower alkoxy, oxo, oxime, lower alkanoyloxy, aryloxy, aryl, benzoyl, a C_4 lactone, a C_4 lactone substituted by a methyl and a hydroxy group, C_3 - C_6 cycloalkyl, or C_3 - C_6 cycloalkyl substituted by hydroxy, lower alkyl, or hydroxyloweralkyl;

R_2 is a methyl group and R_3 is hydrogen, or

R_2 is hydrogen and R_3 is a methyl group, or

R_2 and R_3 are both hydrogen or,

R_2 and R_3 together are a methylene group ($=CH_2$),

R_4 is hydrogen, hydroxy, lower alkoxy or lower alkanoyloxy,

W is oxygen or amino;

X is carbonyl ($C=O$) or methylene (CH_2);

16 Y is oxygen, sulfur, amino, -C(O)O- or -C(O)NH-;
17 Z is biotin, a fluorescent group or a chemiluminescent group; and
18 n_1 and n_2 are independently 1, 2, 3, 4, or 5.

1 2. The compound of claim 1, wherein Z is biotin.

1 3. The compound of claim 1, which is 25-hydroxy-3-
2 aminopropoxyvitamin D₃ biotinamide.

1 4. The compound of claim 1, which is 25-hydroxy-3beta-[(5-
2 biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃

1 5. The compound of claim 1, which is 25-hydroxy-3beta-[(6-
2 biotinamidyl)-hexanamido]-3-aminopropoxyvitamin D₃.

1 6. The compound of claim 1, wherein Z is a fluorescent group.

1 7. The compound of claim 1, which is 25-hydroxy-3beta-
2 aminopropoxyvitamin D₃ fluorescein amide.

1 8. The compound of claim 1, which is 1,25-dihydroxy-3-
2 aminopropoxyvitamin D₃ biotinamide.

1 9. The compound of claim 1, which is 1,25-dihydroxy-3beta-[(5-
2 biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃

1 10. The compound of claim 1, which is 1,25-dihydroxy-3beta-[(6-
2 biotinamidyl)-hexanamido]-3-aminopropoxyvitamin D₃.

1 11. The compound of claim 1, which is 1,25-dihydroxy-3beta-
2 aminopropoxyvitamin D₃ fluorescein amide.

1 12. The compound of claim 1, which is 3-aminopropoxyvitamin D₃
2 biotinamide.

1 13. The compound of claim 1, which is 3beta-[(5-
2 biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃

1 14. The compound of claim 1, which is 3beta-[(6-biotinamidyl)-
2 hexanamido]-3-aminopropoxyvitamin D₃.

1 15. The compound of claim 1, which is 3beta-aminopropoxyvitamin
2 D₃ fluorescein amide.

1 16. The compound of claim 1, wherein R₁ is the side chain of vitamin
2 D₂ or vitamin D₃.

1 17. The compound of claim 1, wherein R₁ is the side chain of vitamin
2 D₂ or vitamin D₃ substituted with at least one hydroxy group at the C₂₃, C₂₄ or C₂₅
3 positions.

1 18. A kit comprising a carrier means having in close confinement
2 therein one or more container means, wherein a first container means contains the
3 compound of claim 1.

1 19. An assay method for the presence of a vitamin D compound in a
2 sample, the improvement comprising using as the labeled vitamin D compound,
3 a labeled compound of claim 1.

1 20. The method of claim 19, wherein Z is biotin.

1 21. The method of claim 19, wherein said labeled compound is 25-
2 hydroxy-3-aminopropoxyvitamin D₃ biotinamide, 25-hydroxy-3beta-[(5-
3 biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃, 25-hydroxy-3beta-[(6-
4 biotinamidyl)-hexanamido]-3-aminopropoxyvitamin D₃, 25-hydroxy-3beta-
5 aminopropoxyvitamin D₃ fluorescein amide, 1,25-dihydroxy-3-
6 aminopropoxyvitamin D₃ biotinamide, 1,25-dihydroxy-3beta-[(5-
7 biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃, 1,25-dihydroxy-3beta-
8 [(6-biotinamidyl)-hexanamido]-3-aminopropoxyvitamin D₃, 1,25-dihydroxy-
9 3beta-aminopropoxyvitamin D₃ fluorescein amide, 3-aminopropoxyvitamin D₃
10 biotinamide, 3beta-[(5-biotinamidyl)pentanamido]-3-aminopropoxyvitamin D₃,
11 3beta-[(6-biotinamidyl)-hexanamido]-3-aminopropoxyvitamin D₃, or 3beta-
12 aminopropoxyvitamin D₃ fluorescein amide.

1 22. The method of claim 19, wherein R₁ is the side chain of vitamin
2 D₂ or vitamin D₃.

1 23. The method of claim 19, wherein R₁ is the side chain of vitamin
2 D₂ or vitamin D₃ substituted with at least one hydroxy group at the C₂₃, C₂₄ or C₂₅
3 positions.

1 24. A solid phase assay method for detecting a vitamin D, its
2 metabolite or analog in a test sample, comprising

3 (a) providing a solid phase support having immobilized thereon a protein
4 or antibody which is capable of binding to the labeled compound of claim 1;

5 (b) contacting said solid phase support with a solution of the labeled
6 compound of claim 1 for a time sufficient to allow binding of the labeled
7 compound to said protein or antibody;

8 (c) washing the solid phase support obtained in step (b) for a time
9 sufficient to remove unbound labeled compound;

10 (d) contacting the solid phase support obtained in step (c) with a liquid
11 test sample suspected of containing a vitamin D, its metabolite or analog for a
12 time sufficient to effect displacement of the labeled compound from said protein
13 or antibody;

14 (e) removing the liquid obtained in step (d); and

15 (f) detecting the presence of the labeled compound in the liquid obtained
16 in step (e);

17 wherein the amount of labeled compound detected in step (f) is directly
18 proportional to the amount of the vitamin D, its metabolite or analog in said test
19 sample.

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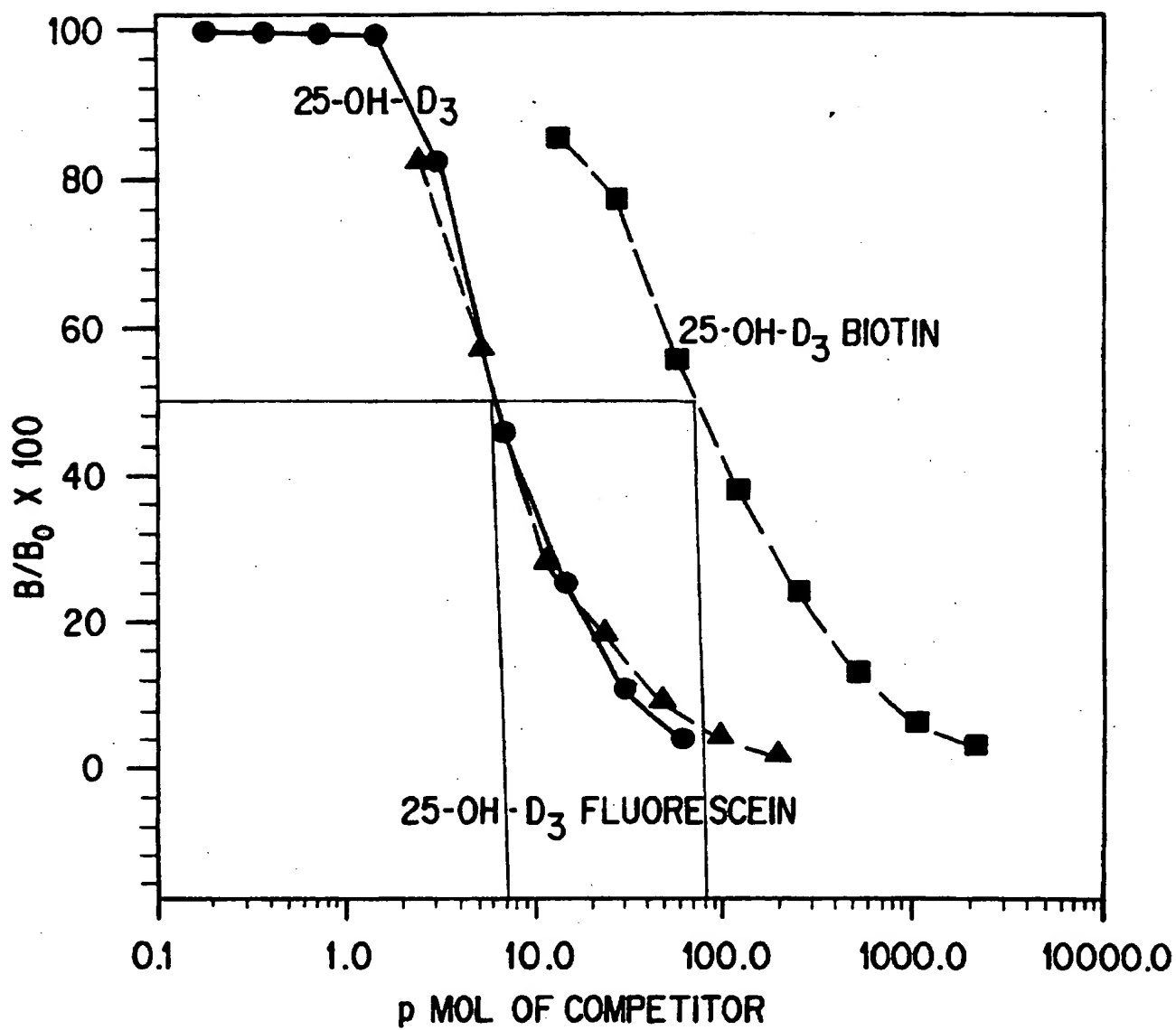


FIG.1

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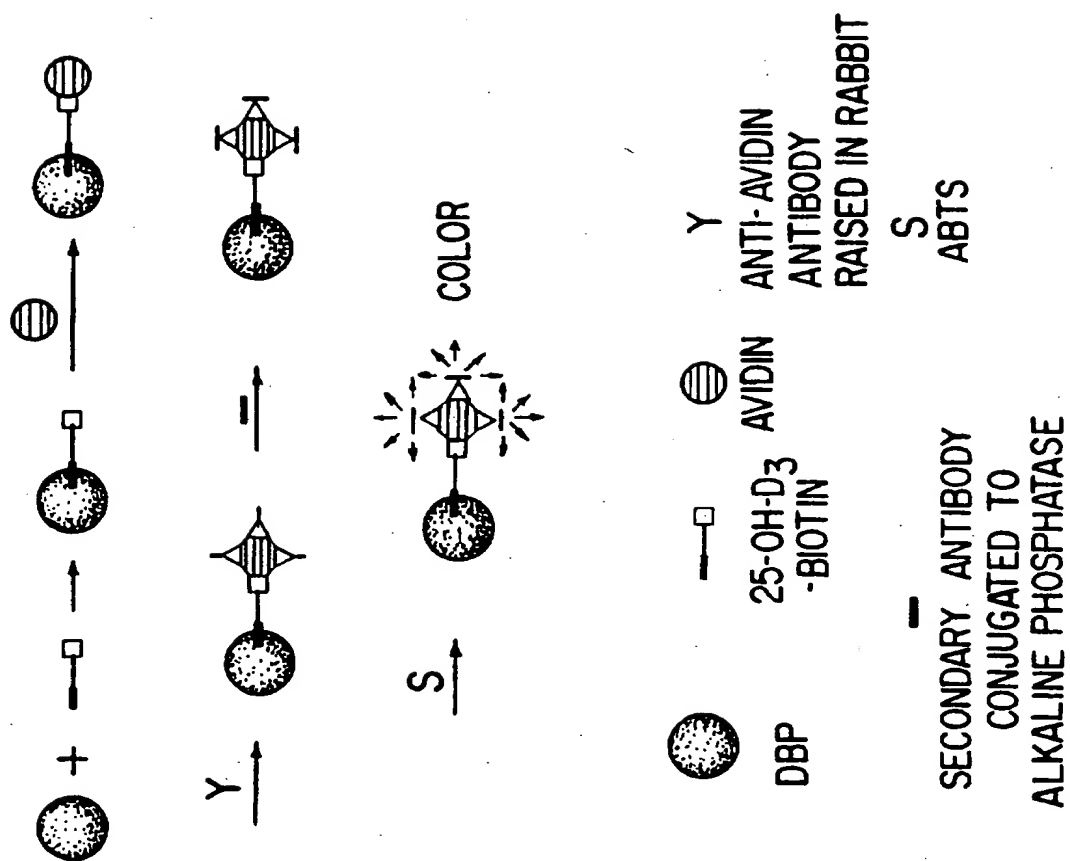


FIG.2

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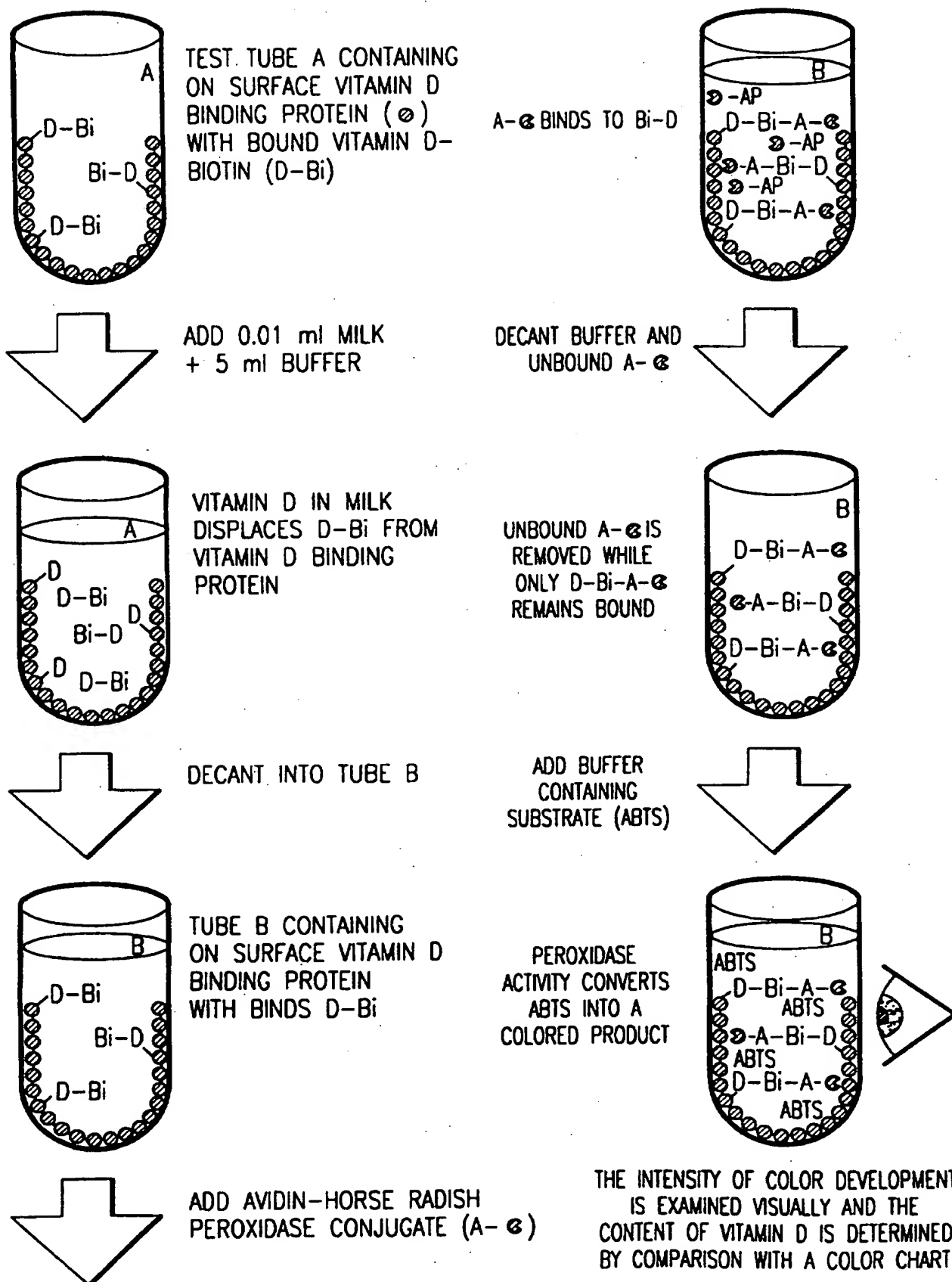


FIG.3

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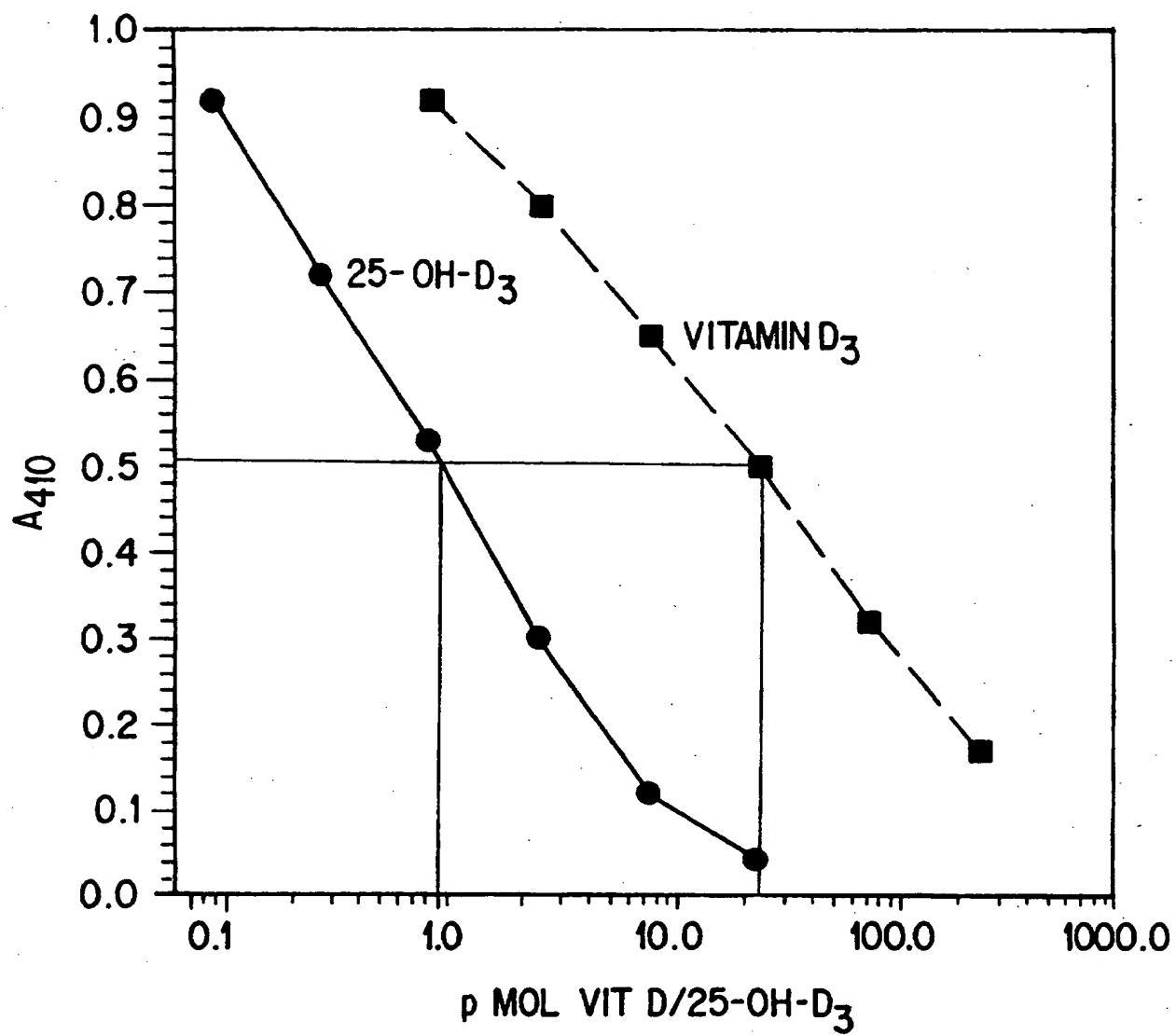


FIG.4

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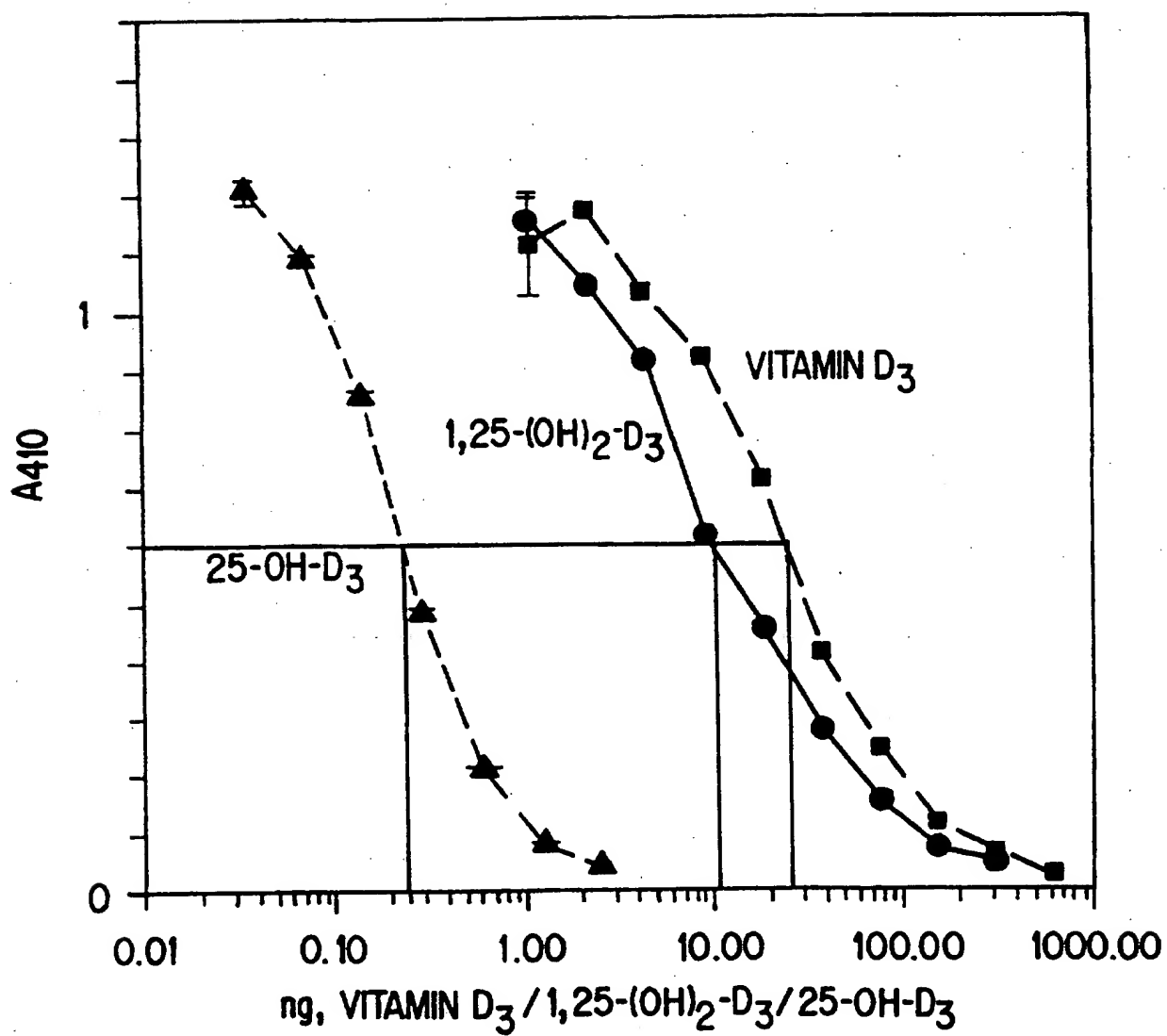


FIG. 5

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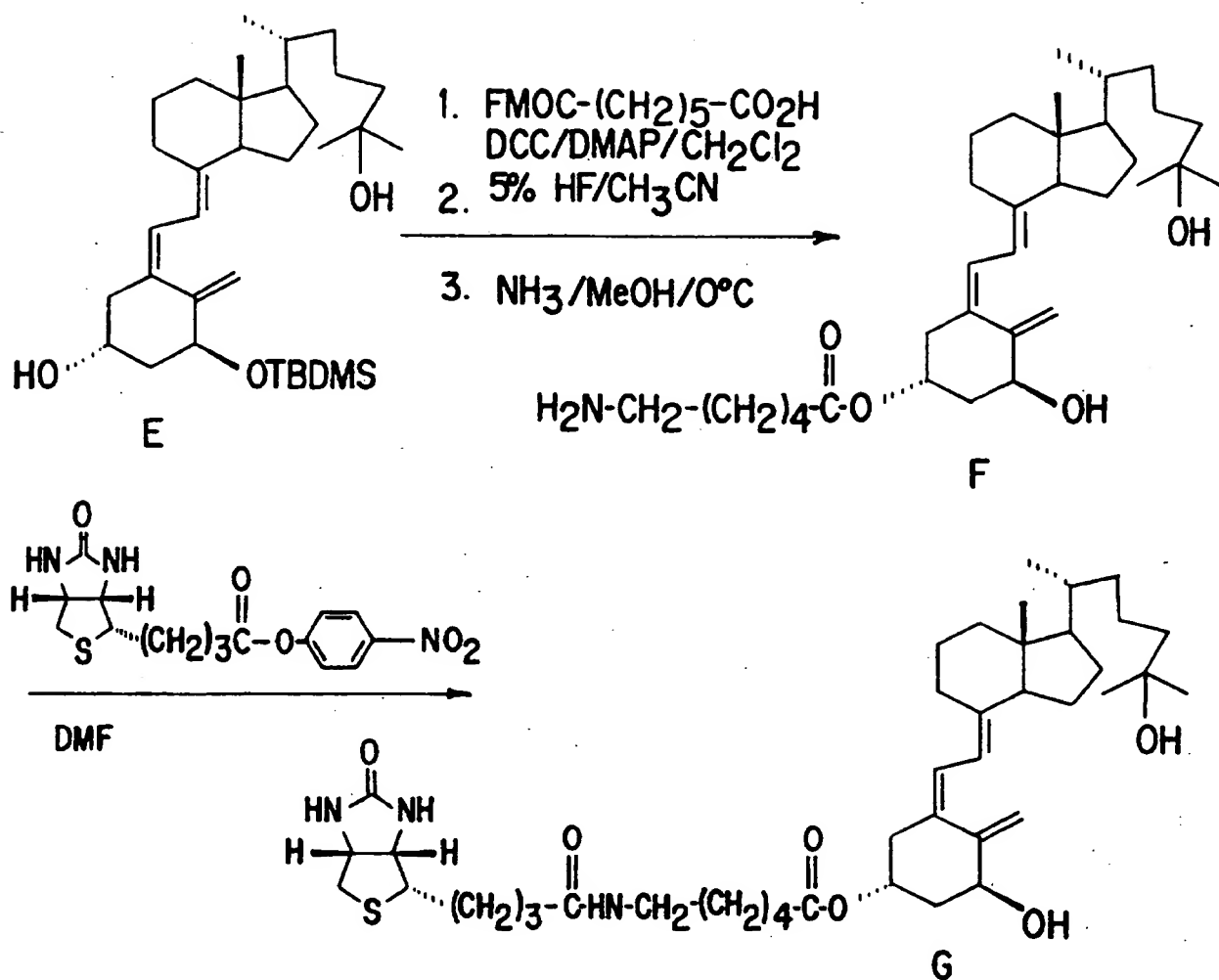


FIG.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20341

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/167, 168; 544/1, 237; 548/303.7, 335.1; 549/227, 265, 510, 511; 552/653

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,933,276 A (BARET) 12 June 1990, see entire document.	1-24
A	US, 5,248,618 A (HACES) 28 September 1993, see entire document.	1-24
A	US 5,430,196 A (DELUCA et al) 04 July 1995, see entire document.	1-24
A	US 5,428,029 A (DORAN et al) 27 June 1995, see entire document.	1-24
A	US 5,411,949 A (NEEF et al) 02 May 1995, see entire document.	1-24

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
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- *O* document referring to an oral disclosure, use, exhibition or other means
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T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z

document member of the same patent family

Date of the actual completion of the international search

28 MARCH 1997

Date of mailing of the international search report

09 APR 1997

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/20341

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 31/59; C07C 401/00; C07D 233/54, 233/56, 233/58, 311/82, 237/32, 321/00, 407/02, 409/02; C07F 5/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/167, 168; 544/1, 237; 548/303.7, 335.1; 549/227, 265, 510, 511; 552/653